

A molecular phylogenetic study of *Clarohilum henningsii* (Mycosphaerellaceae, Fungi) on cassava from Indonesia based on the ITS rDNA sequence

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Abstract

Leaf spot is a common disease of cassava in Indonesia and other tropical countries. The present study aims to determine two isolates isolated from leaf spot of cassava in Indonesia using a molecular phylogenetic analysis based on internal transcribed spacer (ITS) rDNA sequence and morphological examination. The results showed that the two isolates belong to *Clarohilum henningsii*. This study revealed the phylogenetic position of *C. henningsii* from Indonesia, where in the previous studies, it has been reported as *Cercospora cassavae*, *C. manihotis*, *C. henningsii*, or *Passalora henningsii* based on morphological examinations.

Keywords: cassava, ITS rDNA, Indonesia, leaf spot, phylogenetic

Introduction

Cassava brown leaf spot (BLS), caused by *Clarohilum henningsii* (Allesch.) Videira & Crous [syn. *Passalora henningsii* (Allesch.) R.F. Castañeda & U. Braun], is commonly found in many areas where the cassava (*Manihot esculenta* Crantz.) is cultivated. The symptom of this phytopathogen is characterized by having circular lesions, sometimes angular and limited by leaf veins, ± 15 mm diam., brown on upper surface and grey on lower surface with distinct border (Phengsintham *et al.* 2013). Significant yield loss of cassava tuber is possibly related to the reduction of photosynthesis capacity of the plant due to the leaf spots. The current geographical distribution of the BLS disease on cassava includes South America (Brazil, Venezuela), Central America (Nicaragua, Panama), Caribbean (Cuba), Southeast Asia (Brunei Darussalam, Laos, Thailand), East Asia (China), South Asia (India) and South Africa (Farr & Rossman 2020).

In Indonesia, the BLS of cassava has been reported as *Cercospora cassavae*, *C. manihotis*, or *C. henningsii* based on morphological data by van Overeem (1925), with the lack of molecular data. Recently, Videira *et al.* (2017) have been revealed the phylogenetic analysis of genera in the Mycosphaerellaceae. Their results clarified the phylogenetic position of *P. henningsii* into a new cluster. During the survey of leaf spot disease in various plants in Indonesia, we found the BLS symptom on cassava leaves in Cibinong, West Java province.

In this study, we report the morphological characteristics of the causal agent of this BLS symptom and analyse their phylogenetic affinity with other closely related species based on the ITS rDNA sequence.

Materials and methods

Collection sites and morphological examination

Specimens of cassava leaves were collected at cassava plantation in Cibinong Science Center (CSC), Cibinong, West Java province, Indonesia in September 2019. The specimens with brown spot symptoms on leaves were collected during the course of field trips. Fruiting bodies of pathogen on symptomatic leaf were observed using dissecting microscope (OLYMPUS® SZX7, Japan), and detailed morphological characters observation was conducted by compound microscope (OLYMPUS® BX53, Japan). The specimens were prepared by hand sectioning for microscopic examination. Water and Shear's solution were used as mounting media. Thirty conidia, hila, conidiophores, conidiogenous loci and 10 stromata were measured for each specimen. Photograph of microscopic structures were prepared at 400× magnification. Cultures isolated from the specimens have also been deposited at InaCC (Indonesian Culture Collection), Indonesia.

DNA extraction, polymerase chain reaction (PCR) and sequencing

Molecular phylogenetic analysis was conducted to confirm the morphological-based identification, and to elucidate the phylogenetic relationship with similar taxa. Genomic DNA was extracted from 7 d fungal mycelia growth in 5 mL of Potato Dextrose Broth (PDB) using Phytopure™ DNA extraction kit (GE Healthcare, UK) following manufacturer's protocol. Polymerase Chain Reaction (PCR) of the genomic DNA was performed in a 25 mL reaction volume as follow: 12.5 µL of GoTaq® green master mix 2× (Promega, USA), @0.5 µL of forward and reverse primers, 0.5 µL DMSO, 1 µL DNA template, and 10 µL nuclease free water. The primer pairs of ITS5 (forward) (5'-TCCGCTTATTGATATGC-3') and ITS4 (reverse) (5'-TCCGTAGGTGAACCTGCGC-3') (White *et al.* 1990) were used for PCR amplification of the ITS region including 5.8S rDNA. The PCR condition was set as follow: 90 s at 95°C for initial denaturation, followed by 35 cycles of 30 s at denaturation, 30 s at 55°C annealing, 90 s at 72°C extension and 5 min at 72°C for the final extension. PCR products were electrophorized in a 1% (w/v) agarose gel soaked in 1× TAE buffer at 100V for 20 min. 1 kb DNA ladder was used as a marker during the electrophoresis. The gel was soaked in EtBr (ethidium bromide) for 30 min prior to UV light visualization using Gel Doc XR system (Bio-Rad, USA). The PCR products were sent to 1stBASE (Malaysia) for sequencing.

Sequence alignment and phylogenetic analysis

Nucleotide sequences obtained from the ITS5 and ITS4 were examined and refined using Chromas Pro 1.7.5 software (Technelysium Pty Ltd., Australia). Newly ITS sequences of two isolates obtained from cassava were aligned with closely related DNA sequence retrieved from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) using MUSCLE (Edgar 2004) implemented in MEGA 7 (Kumar *et al.* 2016). *Ramichloridium luteum* (NR 119684) (Bakhshi & Zare 2020) was used as an outgroup. Phylogenetic analysis was conducted using the neighbour-joining (NJ) method (Saitou & Nei 1987) implemented in MEGA 7 using Kimura 2-parameter + G + I model as the best evolutionary model for the dataset (Kimura 1980). Maximum composite likelihood was selected as the substitution model for the analysis. Strength of the internal branches of the phylogenetic trees was tested with bootstrap (BS) analysis (Felsenstein 1985) using 1000 replications. Other parameters used in the NJ analysis were selected according to the default standard in the MEGA 7 software. Bootstrap values of

50% or higher were shown. GenBank accession number, sequence name and strain code used in the phylogenetic analysis were showed in Figure 2.

Results

Morphological examination

Clarohilum henningsii (Allesch.) Videira & Crous, Stud. Mycol. 87: 334 (2017) Figure 1a–h.

Description – *Stromata* 35–(50)–60 μm diam. ($n = 10$), ellipsoidal, brown to dark brown, stroma cells angular, 3–(5)–7 μm wide ($n = 13$), smooth. *Conidiophores* 24–(34)–47 \times 3.5–(5)–6 μm ($n = 30$), densely fasciculate, arising from stromata (5–40 per fascicle), not branched, slightly not geniculate, mostly short, cylindrical, 0–2-septate, straight to curved, distance between septa 12–(17)–23 μm ($n = 30$), uniformly pale to slightly olivaceous-brown, smooth. *Conidiogenous cells* 16–(28)–35 \times 3–(4)–5 μm ($n = 14$), terminal, integrated, sympodial, conidiogenous loci small, at the apex, conspicuous, ovoid to oval, 1–2 μm wide ($n = 14$), distinctly darkened. *Conidia* 27–(46)–72 \times 4–(5.5)–7 μm ($n = 30$), solitary, obclavate or cylindrical, 0–6-septate, straight to slightly curved, pale olivaceous brown, smooth, tip subobtuse at the apex, base obconic at the base. *Hila* 0.7–(1.4)–3 μm wide ($n = 30$), thickened and darkened.

Host – *Manihot esculenta*

Material examined – INDONESIA, West Java Province, Cibinong, Cibinong Science Center (CSC), on leaf spot of *M. esculenta*, 1 September 2019, I. Hidayat, (cultures InaCC F1044–InaCC F1045) (GenBank accession number LC565140–LC565141).

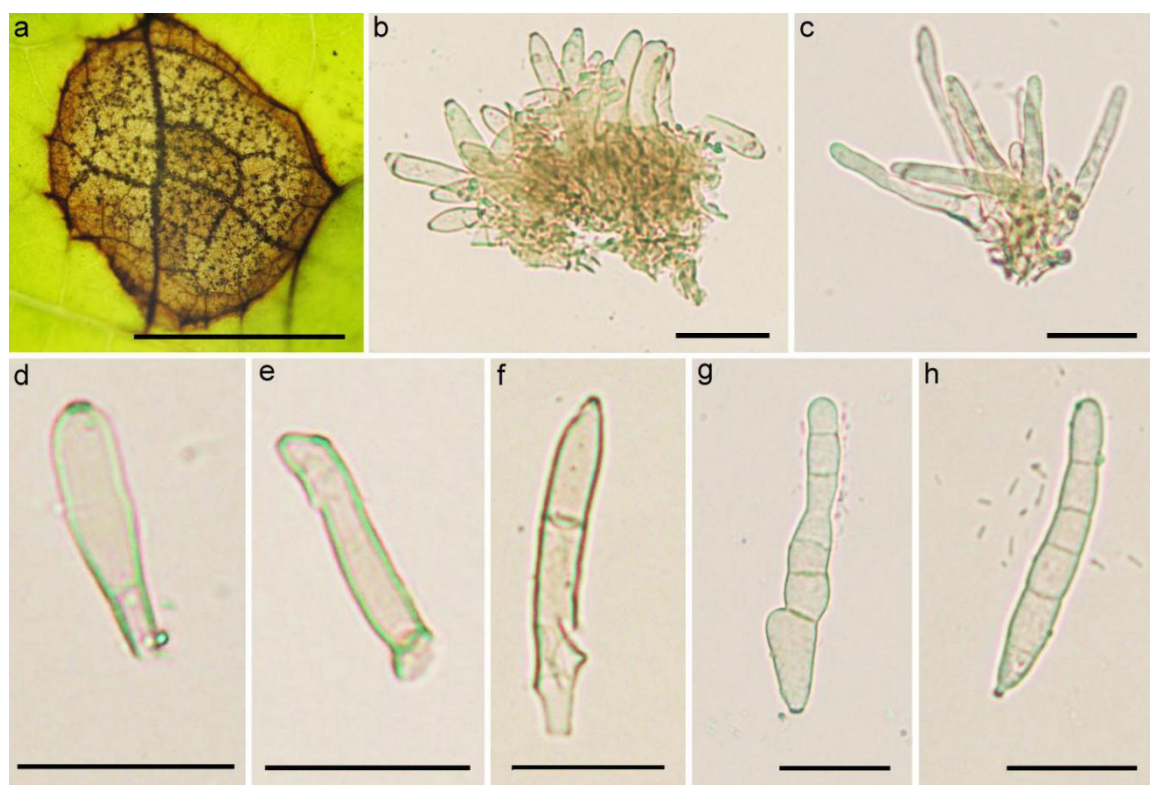


Figure 1. *Clarohilum henningsii* on *M. esculenta*. a Lesion on host leaf (upper surface); b–c stroma with attached conidiophores; d–f conidiophores; g–h conidia. Bars: a = 10 mm, b–h = 20 μm .

Phylogenetic analysis

Based on the phylogenetic tree generated from the NJ analysis, two sequences of cercosporoid fungi isolated from leaf spot of cassava from Indonesia nested the same clade with the type sequence of *C. henningsii* strain CPC 17314 (MF951307) from Laos with 70% BS (Figure 2). The NJ apparently showed that the two sequences from cassava in Indonesia belong to *C. henningsii*.

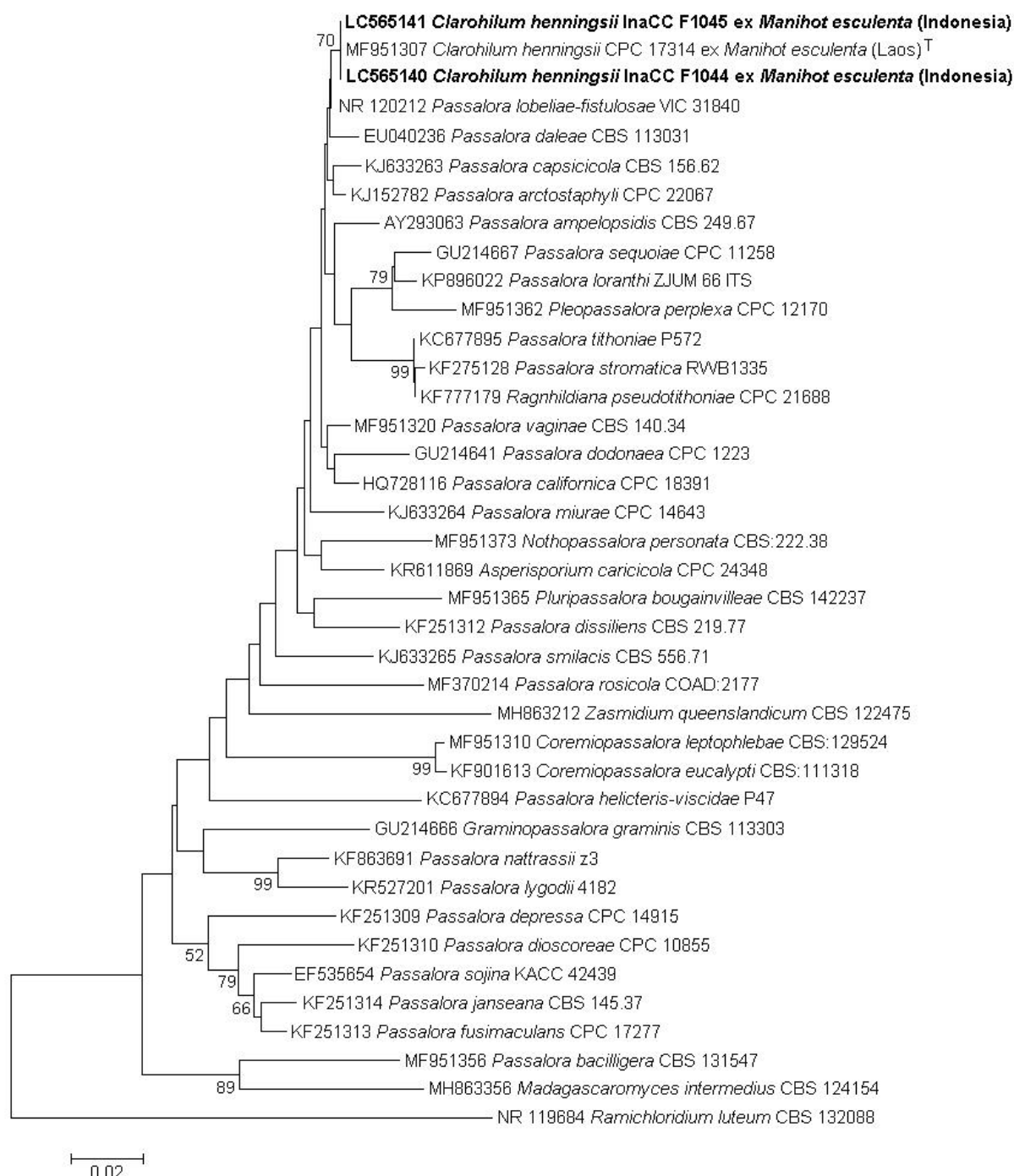


Figure 2. Neighbour-Joining (NJ) tree based on the ITS rDNA sequences showing the relationship of *C. henningsii* from Indonesia with closely related taxa of Mycosphaerellaceae. Bootstrap value > 50% is shown at the nodes.

Discussion

Five species of cercosporoid fungi have been recorded found on leaf spot of *M. esculenta* worldwide (Farr & Rossman 2020). These include *Asperisporium caricae* (Speg.) Maubl., *Cercospora manihobae* Viégas, *Clarohilum henningsii* (Allesch.) Videira & Crous, *Passalora manihotis* (F. Stevens & Solheim) U. Braun & Crous, and *P. vicosae* Crous, Alfenas & R.W. Barreto ex Crous & U. Braun (Index Fungorum 2020). *Clarohilum henningsii* was commonly found causing leaf spot on various plants in subtropical and tropical regions (Farr & Rossman 2020). The morphological examination of the *C. henningsii* specimens from cassava in Indonesia is similar to *C. henningsii* from Laos by having protruding hila at the conidial base. This species was previously described as *P. henningsii* (Chupp 1954, Videira *et al.* 2017). Videira *et al.* (2017) noted that *C. henningsii* differs from *Passalora* s.lat. due to having paler caespituli and conidia with distinctly protruding hila.

Clarohilum henningsii strain from Laos has been well studied by Videira *et al.* (2017). Videira *et al.* (2017) also showed that *C. henningsii* formed a single-strain lineage in the phylogenetic tree of the Mycosphaerellaceae genera based on sequence data of LSU, ITS, and the *rpb2* loci. In the phylogenetic tree, *C. henningsii* is close to *Nothopassalora personata*, but with a low bootstrap support, hence the phylogenetic position was unstable. Morphologically, the conidia of *C. henningsii* showed protruding hila, whereas the conidia of *N. personata* showed less-protruding hila. Therefore, *C. henningsii* was separated from *N. personata* and formed a new genus (Videira *et al.* 2017). In this study, our isolates from necrotic leaves of cassava were confirmed as *C. henningsii* based on the morphological and molecular phylogenetic analyses.

Conflict of interest

The authors state no conflict of interest from this manuscript.

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Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. IH, AH, and IR designed the study; performed research and collected the data; analysed the data; IH, AH, and IR wrote the paper. IH, AH, and IR are the main contributor of this manuscript.

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